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ORIGINAL CONTRIBUTION

Pomegranate and green tea extracts protect against ER stress induced by a high-fat diet in skeletal muscle of mice

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Abstract
Purpose We tested the hypothesis that polyphenol-rich extracts can reduce endoplasmic reticulum (ER) stress induced by a high-fat diet (HFD) in skeletal muscle of mice.
Methods Mice were randomly assigned to four groups receiving during 20 weeks either a standard chow control (CTRL), or a HFD supplemented, or not, with pomegranate (HFD + P) or green tea (HFD + GT) extracts. After the nutritional intervention, mice were killed and gastrocnemius muscles were taken. Proteins and mRNA were measured by Western blot and RT-qPCR, respectively.
Results Body weight gain and visceral fat were higher in HFD, HFD + P and HFD + GT than in CTRL. The markers of the unfolded protein response BiP, XBP1u, XBP1s and ATF4 were higher only in HFD. In HFD + P and HFD + GT, this increase was not observed except for CHOP, which was elevated in all HFD groups. HFD increased also markers of ubiquitin–proteasome pathway, autophagy and oxidative stress, which were kept low in HFD + P and HFD + GT groups.
Conclusion Our data provide evidence for a protective effect of pomegranate and green tea extracts against ER stress, oxidative stress and protein degradation induced by HFD in skeletal muscle. They give arguments for a usefulness of these natural nutritional compounds to fight against cellular dysfunctions related to fat excess.

Keywords High-fat diet · Unfolded protein response · Polyphenols · Protein degradation · Oxidative stress

Abbreviations

ER	Endoplasmic reticulum
UPR	Unfolded protein response
HFD	High-fat diet
EGCG	Epigallocatechin gallate
P	Pomegranate
GT	Green tea

Introduction

Evidence from human and animal studies revealed that excessive lipid accumulation plays an important role in the development of various diseases like obesity, insulin resistance, diabetes and heart failure [1–3]. Lipid excess may impair normal cell signaling, thereby causing cellular dysfunctions [4]. In this context, our team has previously demonstrated that high-sucrose/fat diet activates endoplasmic reticulum (ER) stress in skeletal muscle [5–7]. ER is a key organelle one of the major functions of which is to regulate a variety of post-translational protein modifications. Disruption of ER homeostasis leads to the accumulation of unfolded or misfolded proteins in the ER lumen [8]. To cope with this stress, cells activate a signal transduction, widely known as the unfolded protein response (UPR) [9]. The UPR aims at restoring ER homeostasis by activating three molecules: ATF6 (activating transcription factor 6), IRE1α (inositol-requiring 1α) and PERK (protein kinase R-like ER protein

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kinase). At basal level, these factors associate with the protein chaperone BiP, a member of the heat shock protein70 family. Upon ER stress, unfolded proteins accumulate in the ER and bind to BiP. This binding releases BiP from ATF6, IRE1α and PERK, with subsequent activation of these sensors. The downstream UPR signaling leads to: (1) an inhibition of protein synthesis through the phosphorylation of eIF2α (eukaryotic translation initiation factor 2α), (2) a transcriptional up-regulation of genes involved in protein folding or transport through activation of three transcription factors: ATF4 (activating transcription factor 4), XBP1s (spliced form of X-box-binding protein 1) and ATF6, (3) an apoptosis process mediated by CHOP (C/EBP homologous protein). If excessive or prolonged ER stress cannot be alleviated by UPR, functional homeostasis of the ER is impaired and this can induce an inflammatory state, protein degradation and cell death [8].

Extracts of pomegranate peels and green tea leaves contain high amounts of polyphenols widely known for being beneficial for health [10, 11]. Pomegranate extracts and punicalagin (its major component) have massive anti-diabetic, anti-inflammatory, antioxidant and anti-tumoral properties, both in vivo and in vitro [12, 13]. In addition, consumption of green tea has been associated with decreased risks of obesity, diabetes and hypertension [14]. Epigallocatechin-3-gallate (EGCG) is the most abundant and studied polyphenol of green tea. It has antioxidant properties and prevents apoptosis in various human diseases [15].

Recently, a chemical antioxidant, butylated hydroxyanisole (BHA), was demonstrated to be able to reduce ER stress, to attenuate UPR and to repress the apoptotic process by scavenging oxidants in CHO cells [16]. Furthermore, another study revealed that catechins reduce ER stress in fibroblasts of patients suffering from lysosomal storage disorder [17] and evidences support the idea that green tea polyphenols modulate the ER function in hepatocytes (for review see [18]). Whether green tea and pomegranate extracts are protective against ER stress in skeletal muscle is currently unknown. Accordingly, we hypothesized that pomegranate peel or green tea leaves extracts are able to protect against ER stress induced by a HFD in mice skeletal muscle. We also investigated their influence on cellular processes related to ER stress such as oxidative stress, inflammation, activity of mitogen-activated protein kinases (MAPK) and signaling pathways regulating protein breakdown.

Materials and methods

Diets

Extract of pomegranate peels was purchased from Stiernon S.A. (Ghislenghien, Belgium) and extract of green tea leaves came from Naturex (Avignon, France). Commercial standard laboratory chow and high-fat diet were purchased from SAFE (A04, Augy, France) and Research Diets (D12451, New Brunswick, USA), respectively. Standard chow diet contained 10 % calories from fat, 23 % calories from proteins and 67 % calories from carbohydrates including 4 % calories from sucrose. HFD contained 45 % calories from fat, 20 % calories from proteins and 35 % calories from carbohydrates including 17 % calories from sucrose. Pomegranate or green tea extract (0.5 % w/vol) was integrated to the pellets in HFD diets using a cold extrusion process. This cold extrusion process does not involve exposure to high temperatures that could alter the composition of the used extracts. Table 1 displays the polyphenolic content of pomegranate and green tea extracts included in the diets.

Table 1 Polyphenolic content of pomegranate and green tea extracts

	% of pomegranate extract	% of green tea extract
Total polyphenols content	>30	>98
Punicalagin	>8	
Ellagic acid	>5	
Caffeine		<0.5
Catechins		75
Epigallocatechin-3-gallate		>40

Animals

Twelve-week-old female C57/Bl6J mice (Janvier, France) were housed in cages placed in a controlled environment (22–23 °C, 14/10 h light/dark cycle). Mice were fed ad libitum during 20 weeks. They were divided in four groups: control group (CTRL) fed with the standard laboratory chow ($n = 11$), HFD group fed with the high-fat diet ($n = 11$), HFD + P and HFD + GT groups fed with the high-fat diet supplemented with pomegranate extract or green tea extract, respectively ($n = 11$ for each). Body weight was recorded weekly during the 20 weeks of the nutritional intervention. At the end of the protocol, the mice were kept fasted for 6 h to avoid any confounding effect of the acute food ingestion. Then, the mice were anesthetized with an intraperitoneal injection of a solution (4 ml/kg) containing a mix of ketamine (40 mg/ml) and xylazine (4 mg/kg) to preserve organ perfusion during dissection. The depth of anesthesia was assessed by the absence of eyelid and pedal withdrawal reflexes. The right and left gastrocnemius muscles and the visceral adipose tissues were rapidly removed and immediately frozen in liquid nitrogen. At the end of the procedure, mice were killed by cervical dislocation. Samples were stored at −80 °C until processed. All procedures used in this study were accepted by the committee for ethical practices in animal experiments of

the Université catholique de Louvain. The housing conditions were in accordance with the Belgian Law of April 6, 2010, on the protection of laboratory animals (agreement number: LA-1220548).

Protein extraction and immunoblotting

The muscles were ground in a mortar and homogenized in ice-cold buffer [20 mM Tris, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 % Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM 1,4-dithiothreitol (DTT), and 10 % protease inhibitor cocktail 10X (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were centrifuged at 10,000 *g* for 10 min at 4 °C. Supernatants were immediately stored at −80 °C. Protein concentration was determined using the DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium) with bovine serum albumin as a standard. Western blots were performed as previously described [7]. The following primary antibodies were used (1: 1,000 dilution): BiP, phospho-eIF2α (Ser 51), phospho-Akt (Ser473), Akt, phospho-mTOR (Ser2448), mTOR, phospho-S6 ribosomal protein (Ser235/236), phospho-4E-BP1 (Thr37/46), 4E-BP1, phospho-FoxO1 (Thr24)/FoxO3a (Thr32), FoxO3a, phospho-SAPK/JNK (Thr183/Tyr185), phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), and eEF2 (eukaryotic elongation factor 2) were obtained from Cell Signaling Technology (Leiden, The Netherlands).

After washing, chemiluminescent detection was carried out using an ECL Western blotting kit (Amersham ECL Plus, GE Healthcare, Diegem, Belgium). Pictures were taken with a charge-coupled device (CCD) camera (Gbox, Syngene, The Netherlands). Signal quantification was determined by GeneTool software (Syngene). All results were normalized to eEF2 protein and then expressed relative to the control group.

Oxidative stress assessment

The OxyBlot Protein Oxidation Detection Kit (Millipore) was used to detect the presence of carbonyl groups into protein side chains. Briefly, 50 µg of protein lysates were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reacting with a 2,4-dinitrophenylhydrazine solution for 15 min. Then, the solution was neutralized. The derivatized samples were separated by SDS-PAGE and transferred to a PVDF membrane, and a rabbit anti-DNP primary antibody was used to detect total protein carbonylation.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from gastrocnemius muscle using Trizol reagent according to the manufacturer's protocol (Invitrogen, Vilvoorde, Belgium). The RNA quality and quantity were assessed by Nanodrop® spectrophotometry. First-strand cDNAs were synthesized on 1 µg of total RNA

Table 2 Primer sequences (5′–3′)

Gene	Forward	Reverse
RPL-19	GAAGGTCAAAGGGAATGT	CCTTGTCTGCCTTCAGCTTG
BiP	CTATTCCTGCGTCGGTGTGT	GCAAGAACTTGATGTCCTGCT
CHOP	CCTGAGGAGAGAGTGTTC	CTCCTGCAGATCCTCATAC
ATF4	GAGCTTCCTGAACAGCGAAGTG	TGGCCACCTCCAGATAGTCATC
XBP1u	TGAGAACCAGGAGTTAAGAACACGC	CACATAGTCTGAGTGCTGCGG
XBP1s	TGAGAACCAGGAGTTAAGAACACGC	CCTGCACCTGCTGCGGAC
FOXO1	TCAAGGATAAGGGCGACAGC	GTTCCTTCATTCTGCACTCGAAT
FOXO3	GCTCCCCGGACAAACGGCTC	CAGGCCACTTGGAGAGCTGG
LC3b	ATGCCGTCCGAGAAGACCTT	ATCACTGGGATCTTGGTGGG
p62	GGCATTGAGGTTGACATTGA	GTTTCCCGACTCCATCTGTTCC
Gabarapl1	GAGGACCACCCCTTCGAATATC	CAGTGAGGTCGGAGGGCA
MAFbx	CCATCAGGAGAAGTGGATCTATGTT	GCTTCCCCCAAAGTGCAGTA
MURF-1	ACGACATCTTCCAGGCTGCGAATCC	TCTCGTCTTCGTGTTCCTTGC
NOX-2	CCAGTGAAGATGTGTTCAGCT	GCACAGCCAGTAGAAGTAGAT
NOX-4	GGATCACAGAAGGTCCCTAGCAG	GCGGCTACATGCACACCTGAGAA
IL-6	ACTTCCATCCAGTTGCCTT	GAATTGCCATTGCACAACT
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA

RPL19 ribosomal protein L19, *BiP* binding protein, *CHOP* C/EBP homologous, *ATF4* activating transcription factor 4, *XBP1u* X-box-binding protein 1 unspliced, *XBP1s* X-box-binding protein 1 spliced, *FOXO* forkhead box, *LC3b* protein 1 light chain 3b, *p62/Sqstm1* p62/sequestosome 1, *Gabarapl1* γ-aminobutyric acid receptor-associated protein-like 1, *MAFbx* muscle atrophy F-box, *MURF1* muscle ring finger-1, *NOX* NADPH oxidase isoform, *IL-6* interleukin-6, *MCP-1* monocyte chemoattractant protein-1

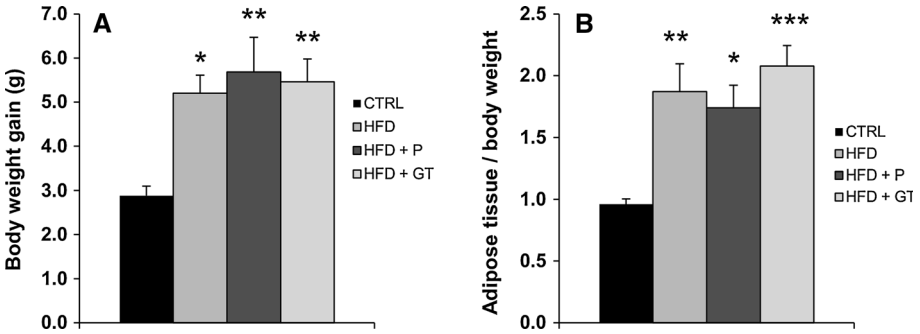

Fig. 1 Body weight gain and visceral fat content. **a** Body weight was measured before and after 20 weeks of HFD to evaluate body weight gain. **b** Perigonadal and perivesical fat content were weighed at the end of 20 weeks HFD and reported to body weight. Results are expressed as the mean ± SEM. *CTRL* control, *HFD* high-fat diet, *HFD + P* high-fat diet plus pomegranate, *HFD + GT* high-fat diet plus green tea ($n = 11$) *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus CTRL

using iScript sDNA synthesis kit (Bio-Rad) with oligo(dT) and random primers. Reverse transcription (RT) products were then diluted in nuclease-free water and kept at −20 °C until use. Real-time PCR experiments were done on a MyIQ2 thermocycler (Bio-Rad) using the following conditions: 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. qPCR mixture contained 4.8 µl IQSybrGreen SuperMix (Bio-Rad), 0.1 µl of each primer (100 nM final) and 5 µl cDNA. Primers sequences are reported in Table 2. The specificity of the amplification was assessed by the melting curve. Each sample was tested in duplicate, and negative controls containing water instead of cDNA template were included in every run. Expression levels of genes of interest were evaluated by qPCR. Analysis was done using the $2^{-\Delta CT}$ method with ribosomal protein L19 (RPL19) as a reference gene.

Statistical analysis

Results are presented as mean ± SEM. The differences between the four groups were tested for significance using a one-way analysis of variance. When significant, the Bonferroni test was used as a post hoc analysis. The significance threshold was set at a P value < 0.05.

Results

Pomegranate and green tea extracts do not protect mice against obesity induced by a high-fat diet

After 20 weeks of nutritional intervention, body weight of CTRL mice increased by 3.0 ± 0.7 g (Fig. 1a). As expected, body weight gain of HFD mice was larger (180 %, $P < 0.05$). Adding pomegranate or green tea extracts to HFD did not change body weight gain (Fig. 1a). In the same way, the HFD groups with or without extracts increased visceral fat content by at least 100 % compared to CTRL mice (Fig. 1b). All together, these results indicate that neither pomegranate nor green tea extract prevents obesity induced by a HFD in mice.

Pomegranate and green tea extracts protect muscle against ER stress induced by a high-fat diet

We next examined the effects of HFD supplemented with pomegranate or green tea extracts on ER stress markers in skeletal muscle. As previously described [5, 7], mRNA of ATF4, CHOP, XBP1u and XBP1s were significantly increased upon HFD (Fig. 2b–e). Here, we also found a BiP mRNA induction after HFD (Fig. 2a). In agreement with our hypothesis, the presence of pomegranate or green tea extracts in HFD counteracted the increase of BiP, ATF4, XBP1u and XBP1s mRNA, whereas CHOP mRNA remained elevated (Fig. 2).

The level of BiP protein was 45 % higher in high-fat fed mice compared to CTRL ($P < 0.05$), whereas it was not affected in the HFD + P and the HFD + GT groups (Fig. 2f). No change was observed in the phosphorylation state of eIF2α between all groups (Fig. 2g).

High-fat diet had no impact on Akt/mTOR signaling pathway activation

ER stress is known to regulate negatively the insulin signaling pathway [19]. Therefore, we decided to investigate the effect of HFD on markers of the Akt/mTOR pathway. The phosphorylation state of Akt was decreased in HFD in comparison with CTRL mice ($P < 0.002$, Fig. 3a). Phosphorylated Akt also tended to decrease in HFD + P and HFD + GT groups but this difference was not significant compared to CTRL group (Fig. 3a). The phosphorylation state of mTOR, rpS6 and 4E-BP1, proteins downstream in the signaling, was not

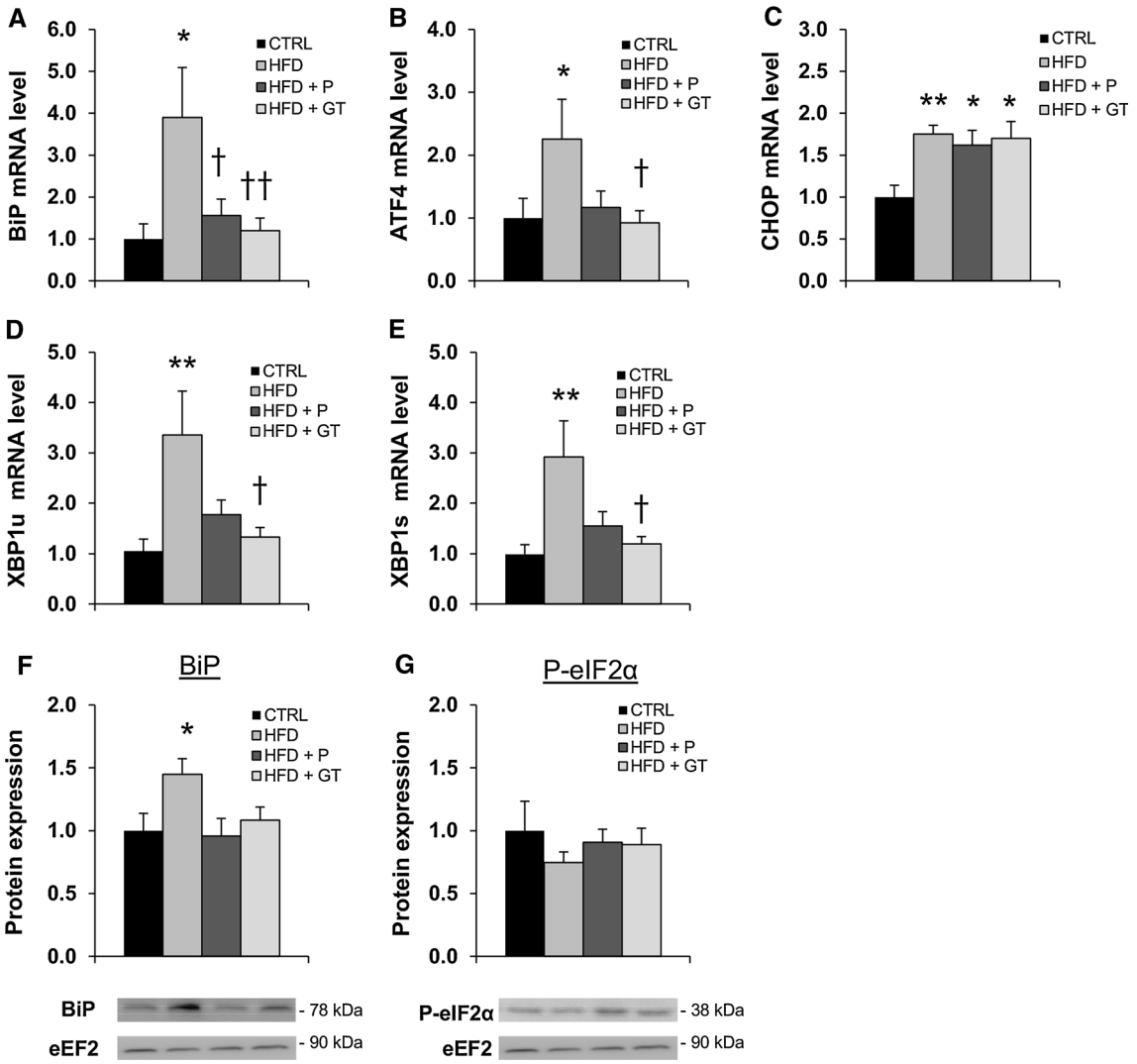

Fig. 2 Endoplasmic reticulum stress markers. mRNA level of **a** BiP, **b** ATF4, **c** CHOP, **d** XBP1u, and **e** XBP1s in gastrocnemius muscle. Protein expression of **f** BiP and phosphorylation state of **g** eIF2α in gastrocnemius after 20 weeks HFD, HFD + P or HFD + GT. Results are expressed as the mean ± SEM. A value of 1 was arbitrarily assigned to the control conditions to which the HFD, HFD + P or HFD + GT values were reported and expressed as foldbasal. *CTRL* control, *HFD* high-fat diet, *HFD + P* high-fat diet plus pomegranate, *HFD + GT* high-fat diet plus green tea ($n = 11$) $^{*}P < 0.05$; $^{**}P < 0.01$ versus CTRL. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus HFD

changed even if there was a systematic tendency to decrease under HFD (Fig. 3b–d). These results indicate that HFD inhibits Akt but this inhibition is not passed on downstream proteins known to regulate protein synthesis, namely mTOR, rpS6 and 4E-BP1.

Pomegranate and green tea extracts prevent the activation of protein degradation signaling pathways induced by a high-fat diet

Akt prevents the nuclear translocation of FoxO3a and FoxO1 by phosphorylating them at multiple sites [20]. We measured the phosphorylation state of FoxO3a and FoxO1 at Thr32/Thr24. They were decreased with HFD ($P = 0.013$, Fig. 4a, b), but remained totally unaffected when pomegranate or green tea extracts were added to HFD. The total form of FoxO3a was unchanged in all conditions (Fig. 4c). To see if a regulation at the transcriptional level occurred for FoxO1 and FoxO3, we measured their transcript level. The mRNA level of FoxO1 was increased in the HFD group, but not with pomegranate or green tea (Fig. 4d). FoxO3a mRNA was also increased by HFD. This increase was slightly reduced in HFD + P and HFDP + GT groups, but without reaching the statistical threshold of significance in any condition (Fig. 4e).

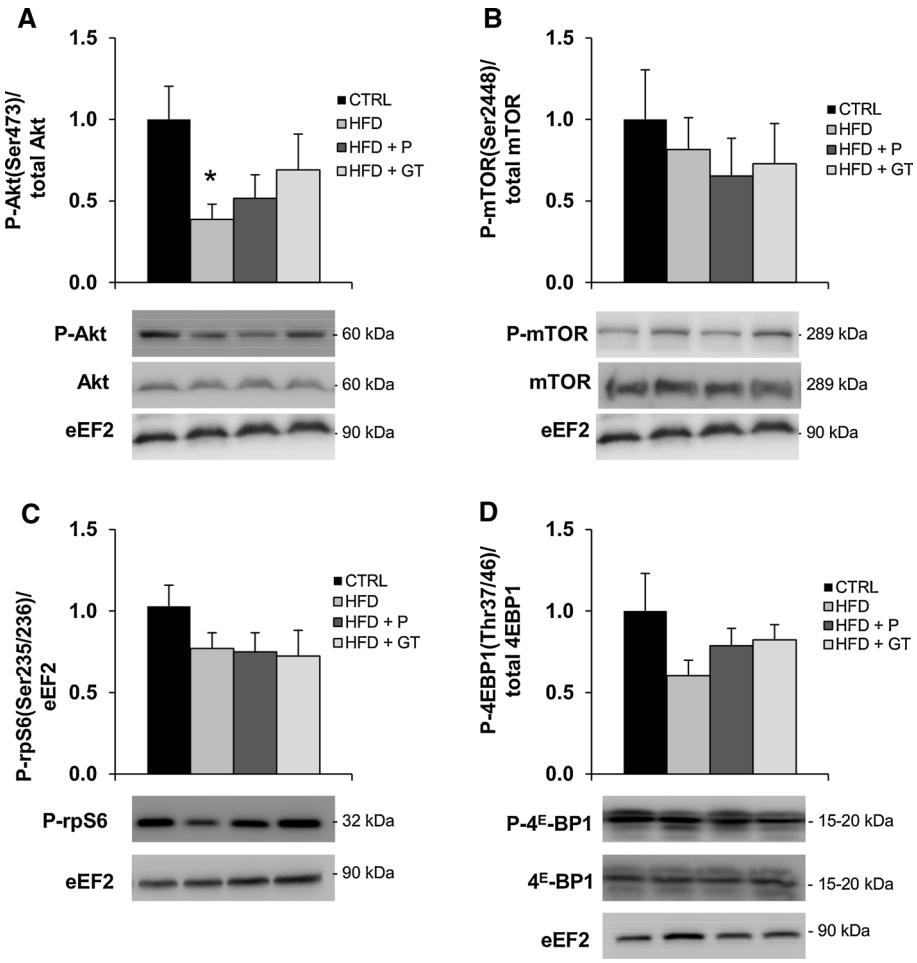

Fig. 3 Protein synthesis signaling pathway. Western blot analysis of gastrocnemius protein extracts from mice fed with standard (CTRL) or high-fat diet (HFD) supplemented in pomegranate (HFD + P) or green tea (HFD + GT). Protein extracts were immunoblotted for the expression and/or phosphorylation of **a** Akt, **b** mTOR, **c** rpS6 or **d** 4E-BP1. Quantification is expressed relative to eEF2 levels. Results are expressed as the mean ± SEM. A value of 1 was arbitrarily assigned to the control conditions to which the HFD, HFD + P or HFD + GT values were reported and expressed as foldbasal ($n = 11$) *$P < 0.05$ versus CTRL

MAFbx and MURF1 are muscle-specific ligases, which are believed to be the rate-limiting steps of protein degradation through the ubiquitin–proteasome system [21]. FoxOs is among the major transcription factors that regulate MAFbx and MURF1 [20]. In comparison with the CTRL group, MAFbx and MURF1 were increased with HFD, but not in HFD + P and HFD + GT mice (Fig. 4f, g). BNIP3, BNIP3L and p62, three markers of the autophagy–lysosomal pathway, were also increased after HFD (Fig. 4h–j, $P = 0.04$, $P = 0.014$ and $P = 0.016$, respectively). HFD enriched with pomegranate and green tea extracts reduced these effects (Fig. 4h–j). Furthermore, even if the differences were not significant, LC3b and Gabarapl-1 showed the same tendency (Fig. 4k, l). Taken together, these results indicate that ubiquitin proteasome and autophagy-related genes were induced by a HFD and these changes can be prevented by pomegranate or green tea extracts.

Pomegranate and green tea extracts prevent oxidative stress induced by a high-fat diet

ER stress seems to be intimately related to the activation of oxidative stress [22, 23]. To test this hypothesis, we decided to measure various oxidative stress markers. NOX2 and NOX4, two members of the NADPH oxidase family, were up-regulated in the HFD in comparison with the CTRL group (Fig. 5a, b, $P = 0.003$ and $P = 0.027$, respectively), whereas pomegranate or green tea extracts prevented the increase after HFD.

The protein carbonyls were more abundant in the HFD than in the CTRL group (Fig. 5c, d). This difference was abolished in the HFD + P and the HFD + GT groups (Fig. 5c, d). Our results confirm that pomegranate and green tea extracts protect against oxidative stress induced by a HFD. Nevertheless, the green tea extract seems to have higher antioxidant properties than the pomegranate extract since the repression of NOX2, NOX4 and proteins carbonyls was systematically greater.

High-fat diet does not increase MAPK and inflammation markers in skeletal muscle

Beside oxidative stress, other cellular stresses, among which inflammation, could be related to the induction of ER stress [24]. Therefore, we decided to evaluate the

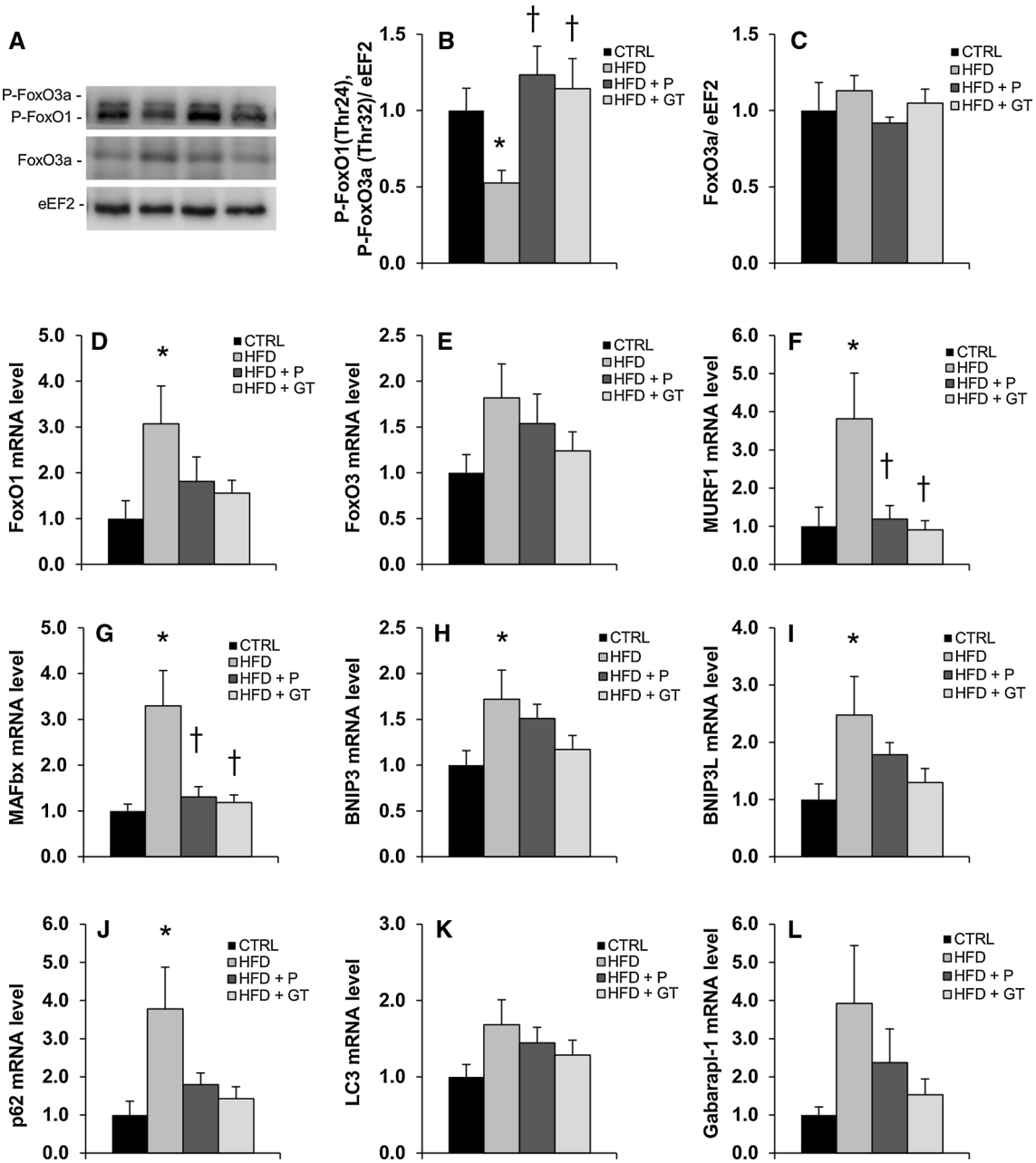

Fig. 4 Protein degradation signaling pathways. **a** Western blot analysis of gastrocnemius protein extracts from mice fed with standard (CTRL) or high-fat diet (HFD) supplemented in pomegranate (HFD + P) or green tea (HFD + GT). Protein extracts were immunoblotted for the expression of FoxO3a and the phosphorylation of both FoxO1 and FoxO3a. **b** Quantification of phospho-protein normalized to eEF2 level. **c** Quantification of FoxO3a protein level expressed relative to eEF2 level. **d–l** mRNA level of FoxO1, FoxO3a, MURF1, MAFbx, BNIP3, BNIP3L, p62, LC3 and Gabarapl-1. Results are expressed as the mean ± SEM. A value of 1 was arbitrarily assigned to the control conditions to which the HFD, HFD + P or HFD + GT values were reported and expressed as foldbasal ($n = 11$) $^{*}P < 0.05$ versus CTRL, $^{\dagger}P < 0.05$ versus HFD

activation of the MAPK (Mitogen-activated protein kinases) pathway and IL-6 and MCP-1 mRNA levels. Neither the levels of IL-6 and MCP-1 mRNA (Fig. 6a, b), nor the phosphorylation state of ERK1/2, p38 or SAPK/JNK (Fig. 6c–e) were changed by HFD. These results suggest that, in our conditions, inflammation and MAPK are not contributing to ER stress.

Discussion

The main findings of the present study are that natural compounds such as pomegranate and green tea extracts protect muscle against ER stress, activation of the protein degradation pathways and oxidative stress induced by a 20-week HFD in mice.

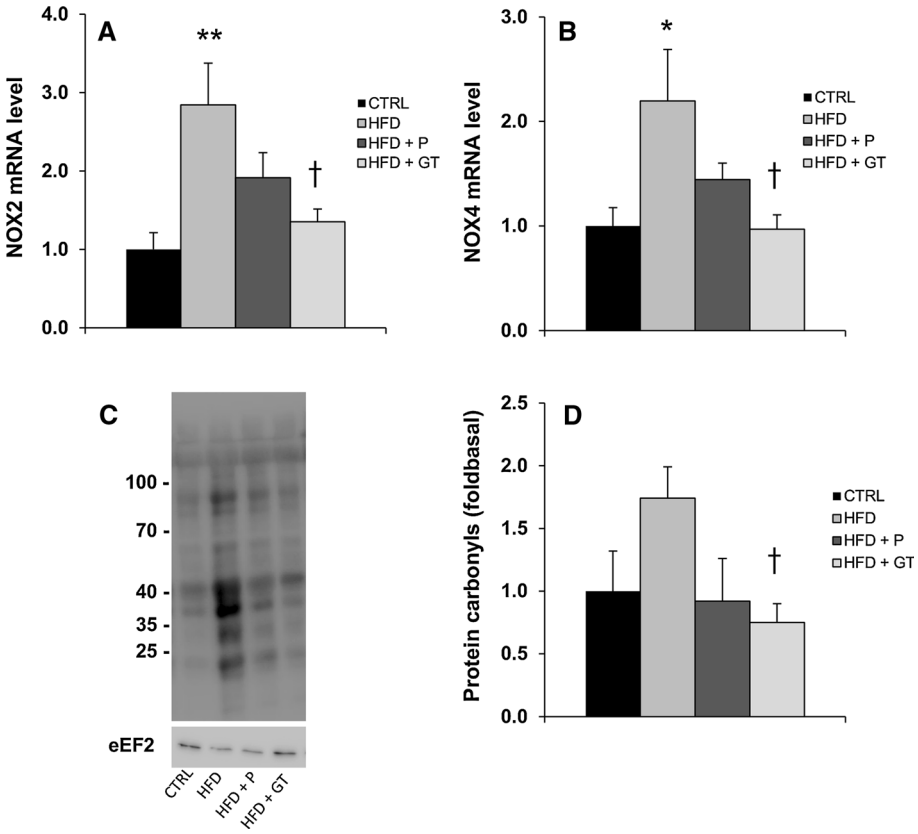

Fig. 5 Oxidative stress markers. mRNA level of **a** NOX2 and **b** NOX4. Results are expressed as the mean ± SEM. *CTRL* control, *HFD* high-fat diet, *HFD + P* high-fat diet plus pomegranate, *HFD + GT* high-fat diet plus green tea. **c** Representative Western blots showing levels of protein carbonylation. **d** Quantification of protein carbonylation relative to eEF2 level. Results are expressed as the mean ± SEM. A value of 1 was arbitrarily assigned to the control conditions to which the HFD, HFD + P or HFD + GT values were reported and expressed as foldbasal ($n = 11$) $^{*}P < 0.05$; $^{**}P < 0.01$ versus CTRL, $^{\dagger}P < 0.05$ versus HFD

Pomegranate and green tea extracts do not protect mice against HFD-induced obesity

In human studies, the typical dose used for pomegranate extracts is about 1 g/day [25–27] and between 300 mg and 4 g/day for green tea extracts [28, 29], which corresponds to 5–65 mg/kg body weight/day. Considering species differences, this corresponds roughly to 500 mg of extracts/kg body weight/day in mice [30, 31]. Based on the content of pomegranate or green tea extract in the pellets (0.5 % w/v) and the amount of pellets ingested (average 3 g/day) in the present study, we calculated that the ingested dose was 600 mg of extracts/kg body weight/day, a dose compatible with the aforementioned human studies.

Neither pomegranate, nor green tea supplement prevents obesity in mice after 20-week HFD. Indeed, mice fed with HFD, HFD + P or HFD + GT showed a similar body weight gain and visceral adipose tissue content. However, all the experimental results reported in the literature do not support the same conclusion. Lei et al. [32] observed anti-obesity effects of a pomegranate leaf extract after a 6-week HFD. Vroegrijk et al. [33] reported also that feeding mice during 12 weeks with a HFD supplemented with 1 % pomegranate seed oil resulted in a decreased body weight and a reduced fat mass content. In the same way, as in the present study, Neyrinck et al. [34] were not able to demonstrate any significant protective effect on body weight gain of a pomegranate peel extract added to food or water during 12 or 20 weeks HFD. As we used the same extract as in Neyrinck et al. [34], the different results found in the literature can likely be explained by the composition of the extracts. Indeed, Vroegrijk et al. [33] used a pomegranate seed oil rich in punicic acid, whereas the two main polyphenols of our pomegranate peel extract were punicalagin and ellagic acid [12].

The explanation is less obvious for the divergent results reported with the green tea extracts. Recently, Cunha et al. [35] reported that incorporation of green tea in water attenuated obesity induced by a 8-week HFD. This observation confirmed previous results obtained on the basis of the incorporation of 0.5 % green tea extract directly into HFD [36]. In this last study, green tea extract enriched with 81.3 % polyphenols was used [35] while our extract was enriched with 75 %. The duration of the experiment was also different (8 weeks vs 20 in this study) as well as the gender of the animals (males vs females in this study). Our mice were weighted every week since the beginning of the experiment and we never detected any difference in the body weight gain between the three HFD groups. Composition of the extracts may not explain this difference because all are rich in catechins, especially epigallocatechin gallate, a flavonoid known to reduce diet-induced

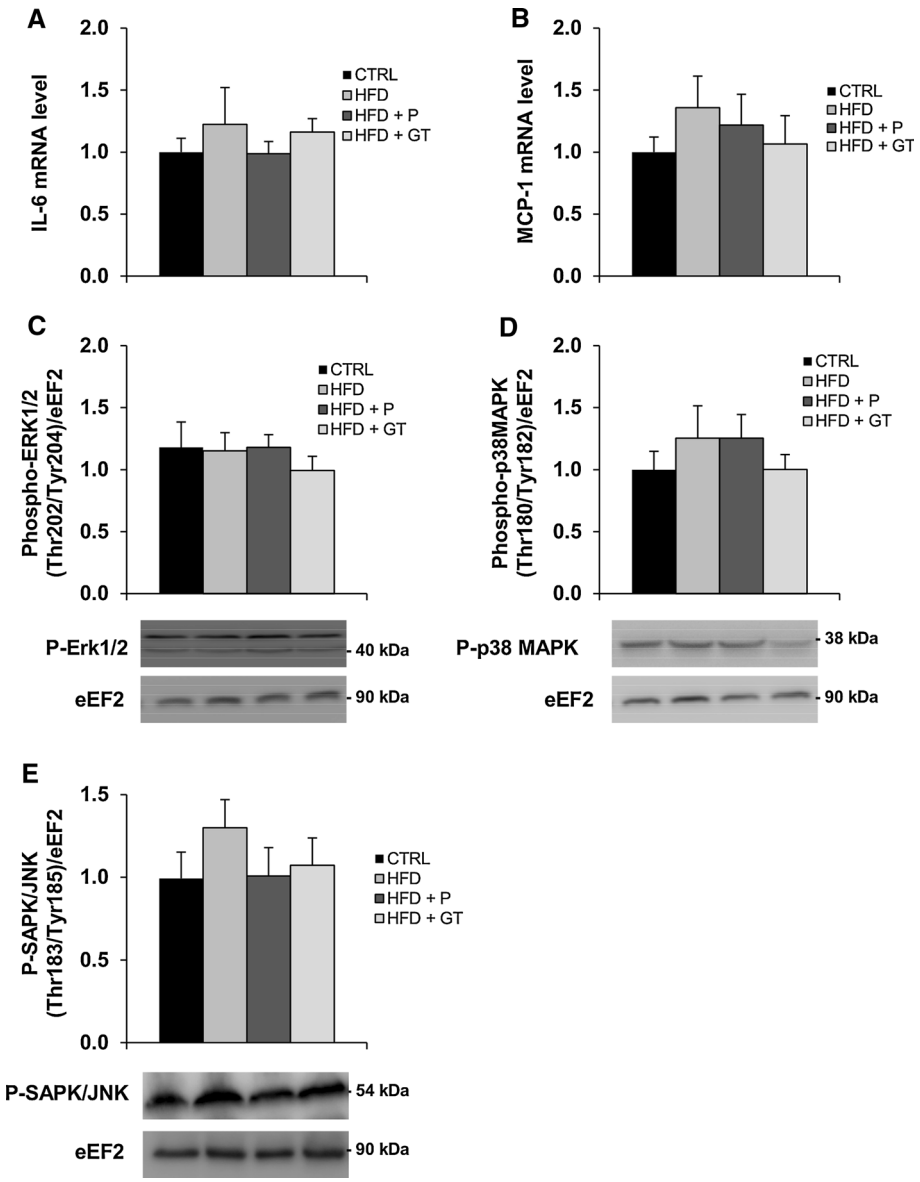

Fig. 6 Inflammation markers. mRNA level of **a** IL-6 and **b** MCP1. Phosphorylation of **c** ERK1/2, **d** p38 and **e** SAPK/JNK in gastrocnemius muscle of mice fed with standard (CTRL) or high-fat diet (HFD) supplemented in pomegranate (HFD + P) or green tea (HFD + GT). Results are expressed as the mean ± SEM. A value of 1 was arbitrarily assigned to the control conditions to which the HFD, HFD + P or HFD + GT values were reported and expressed as foldbasal ($n = 11$)

obesity in mice or humans [37]. It is unlikely that the small difference in polyphenol content may explain the divergent results. Therefore, we may not exclude a gender-specific response. Definitely, further studies are needed to identify the experimental conditions in which green tea has, or not, anti-obesity properties.

Pomegranate and green tea extracts protect muscle against ER stress induced by a high-fat diet

Confirming results previously acquired in our laboratory, the HFD protocol applied in the present study increased several markers of ER stress in mice skeletal muscle as well [5–7]. The main originality of the present study is to evidence that supplementation in pomegranate or green tea extracts repressed the increase of BiP, ATF4, XBP1s and XBP1u induced by a HFD. Since BiP is the key chaperone for activating cell response to ER stress [38], we may conclude that ER stress occurs in skeletal muscle of mice fed with HFD but not in muscle of those fed with HFD + P or HFD + GT.

XBP1u is a member of the ER-stress-inducible gene family, transcriptionally regulated by ATF6 [39]. During ER stress, IRE1α is responsible for the splicing of XBP1u, which leads to the formation of XBP1s. The results regarding XBP1u and XBP1s suggest that both ATF6 and IRE1α pathways are likely implicated in the protection of pomegranate and green tea extracts against ER stress induced by a HFD in muscle.

Various stressors including ER stress and oxidative stress activate ATF4 [40]. Thus, ATF4 must be more

considered as a marker of the integrated stress response than a specific marker of the UPR. ATF4 is translationally regulated by eIF2α, a downstream target of PERK. In this study, we did not measure the protein expression of ATF4, but well the phosphorylation state of eIF2α which remained unchanged in all conditions, suggesting that the PERK pathway is not activated by HFD. Nevertheless, the activation of PERK depends on the acute nutritional status [41] and the animals used in the present experiment were killed after a fasting period of 6 h. Thus, we may not exclude that we missed a transitory activation of the PERK pathway. At the transcriptional level, ATF4 is regulated by nuclear factor-like 2 (Nrf2), which is sensitive to oxidative stress [42]. ATF4 mRNA was clearly increased after HFD, but not after HFD + P and HFD + GT, giving arguments for a protective mechanism based on antioxidant properties of the extracts used in this experiment (see below).

CHOP did not follow the same pattern of regulation as the other UPR markers. CHOP is positively regulated by ATF4, ATF2 and ATF6 and negatively regulated by ATF3 at the transcriptional level [43]. Since ATF4 is not always transcriptionally and translationally regulated in the same way [44] and because the ATF6 activity was not measured in this study, it would be hazardous to speculate about CHOP regulation in our experimental conditions. In any case, CHOP mRNA increased after HFD and the polyphenol-rich extracts had none protective effect. This conclusion may certainly not be extended to all polyphenol-rich extracts since grape seed proanthocyanidin extracts have been showed to alleviate partially ER stress in diabetic rats by decreasing CHOP expression in skeletal muscle [45].

Figure 7 summarizes the UPR signaling pathways activated by HFD and the mechanism of action of pomegranate and green tea.

Effects of pomegranate and green tea extracts on protein synthesis and degradation signaling pathways after a HFD

HFD and chemically induced ER stress are known to independently impair the Akt/mTOR signaling pathway in muscle [46, 47]. Therefore, we wanted to test if the pomegranate and green tea extracts associated with HFD were able to abolish this inhibition. HFD seemed to decrease the phosphorylation state of the major intermediates of this protein synthesis regulatory pathway namely Akt, mTOR, rpS6 and 4E-BP1. Adding polyphenol-rich extracts to HFD did not reverse this general tendency except for Akt, which was less dephosphorylated. This indicates that ER stress is likely not a major actor in HFD-induced inhibition of mTOR and its downstream targets.

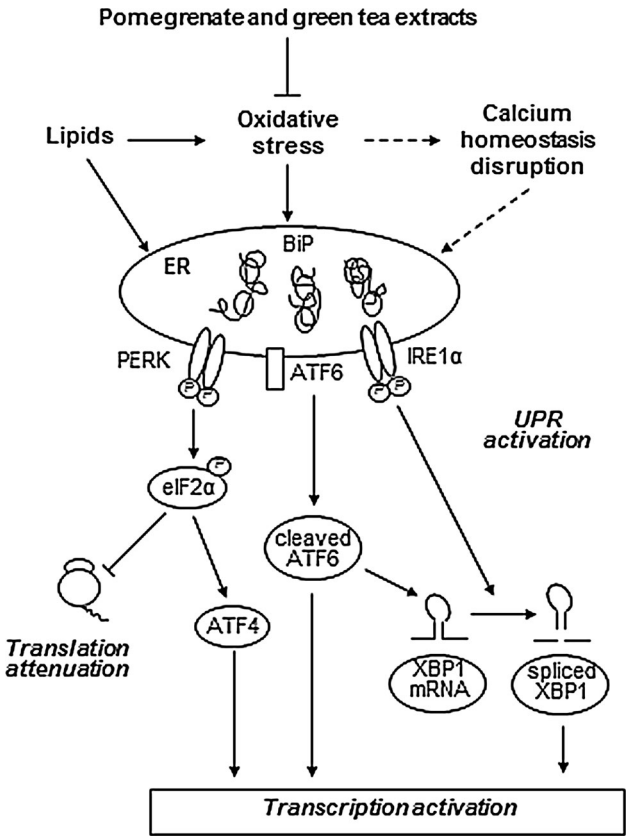

Fig. 7 Signaling pathways of the unfolded protein response activated by high-fat diet and mechanism of action of pomegranate and green tea extracts. The *dashed arrays* show pathways not investigated in the present study

In a previous study, we found an impaired glucose tolerance and the presence of ER stress in mice after a similar HFD [7]. Glucose tolerance test was not performed in the present experiment, but the same pomegranate extract used in a previous study did not reveal any protective effect on glucose intolerance [34]. However, other studies revealed an improvement of glucose tolerance, due to green tea extract supplementation, in rats or mice fed with a HFD [48, 49]. Proanthocyanidins, another polyphenol, also ameliorated glucose intolerance, by regulating ER stress, in pancreas of rats fed with a HFD [50]. All together, these results are compatible with the idea that ER stress impairs glucose tolerance, which could be partially restored by some polyphenol-rich extracts.

Evidence strongly suggests that protein degradation pathways such as the ubiquitin–proteasome and autophagy–lysosome systems are activated by ER stress, probably for removing mis- and unfolded proteins [51]. Therefore, we analyzed several specific markers for those proteolytic pathways. FoxO1 and FoxO3a are transcription factors, the activity of which is mainly regulated by their phosphorylation state [52]. Interestingly, phosphorylation of FoxO1/FoxO3a

was drastically decreased after HFD, whereas it was significantly increased in HFD + P and HFD + GT compared to CTRL. This clearly suggests that pomegranate and green tea extracts prevent the nucleus translocation of FoxO3a after a HFD in gastrocnemius muscle. FoxO3a regulates the transcription of the two main muscle-specific ligases, namely MURF1 and MAFbx [20]. Accordingly, we found that HFD increased MURF1 and MAFbx mRNA. This observation supports an up-regulation of the ubiquitin–proteasome system and is consistent with a recent study reporting a higher level of ubiquitinated proteins in muscle after HFD [53]. Moreover, the increase of MURF1 and MAFbx was largely repressed by pomegranate or green tea extracts. To the best of our knowledge, this is the first study showing that a punicalagin-rich pomegranate extract is able to protect against the increase of muscle-specific ligases during HFD. Our results are in agreement with a study in which epigallocatechin-3-gallate, the major component of our green tea extract, attenuated skeletal muscle atrophy caused by cancer cachexia by inhibiting MAFbx and MURF1 expression [54]. The green tea extract used in the present study completely blunted the up-regulation of ligases in a HFD model.

Since FoxO3a is also implicated in the regulation of the autophagy–lysosome system, we decided to measure autophagy-regulatory (BNIP3, BNIP3L) and autophagy-regulated genes (p62, LC3b or Gabarapl-1) [55, 56]. The general tendency was in favor of an increase of these genes after HFD, whereas the addition of polyphenol-rich extracts tended to attenuate the effect.

All these data indicate that HFD activates protein degradation systems through the Akt/FoxO pathway and pomegranate and green tea extracts have potent preventive properties against this activation.

Do pomegranate and green tea extracts regulate ER stress and protein degradation systems through reduced oxidative stress and inflammation?

Oxidative stress initiates and contributes to the induction of ER stress [57] and autophagy [58]. Consistent with a recent report [53], protein carbonyls were increased in muscle after a HFD. This increase was associated with an elevation of NOX2 and NOX4 (NADPH oxidase isoforms) mRNA levels, confirming the presence of an oxidative stress in skeletal muscle after HFD. Green tea extract has antioxidant effects in vivo [59], and some studies have revealed similar properties for pomegranate peel extract [13]. Here, we confirmed that these two extracts have antioxidant effect in muscle since they blocked the NOX2 and NOX4 induction and reduced the level of protein carbonyls due to HFD. These antioxidant properties of our polyphenol-rich extracts might explain the prevention of ER stress in muscle of mice treated with pomegranate or green tea extracts.

Other cell stresses including inflammation are often linked to ER stress in various tissues of mice upon HFD [60]. Previous work from our laboratory demonstrated that 6 weeks of HF feeding clearly increased plasma levels of cytokines such as IL-6 or MCP-1 and activated IKK, a protein kinase upstream of NFkB. These increases were correlated with an activation of ER stress. However, in this longer experiment, no change was observed in inflammation markers and the phosphorylation state of the MAPK was not affected by HFD in skeletal muscle, confirming previous results of our laboratory obtained with a similar duration of HF feeding [7]. Furthermore, the mRNA levels of IL-6 and MCP-1 were not significantly changed between HFD and CTRL groups. Taken together, these results suggest that inflammation was not increased after a long-term period of HF feeding in mice.

Furthermore, in the HFD used in the present experiment, the sucrose content was higher than in chow (17 vs 4 kcal %), but far from the 60–70 kcal % usually used in the high-sucrose diets [61, 62]. Therefore, it is likely that ER stress, induction of markers involved in the protein degradation pathway and oxidative stress observed in mice fed with a HFD is mostly due the fat content of the diet even if we may not rule out a partial indirect contribution of sucrose.

In conclusion, pomegranate and green tea extracts are able to protect against ER stress induced by HFD in skeletal muscle of mice, although they do not prevent fat accumulation. They also attenuate oxidative stress, activation of the ubiquitin–proteasome pathway and up-regulation of genes implicated in autophagy regulation. By this way, we have demonstrated that some natural extracts are able to protect skeletal muscle against cellular stresses induced by a HFD even if they are not sufficient to prevent obesity. All these processes are closely inter-related and can contribute to a protective action of pomegranate and green tea extracts against ER stress and muscle protein degradation in obese patients.

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Conflict of interest The authors have declared no conflict of interest.

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